

FORM PTO-1390 (Modified)
(REV 11-98)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

41934/23838

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/673785

INTERNATIONAL APPLICATION NO.
PCT/GB99/01211INTERNATIONAL FILING DATE
21.04.99PRIORITY DATE CLAIMED
22.04.98

TITLE OF INVENTION

PEPTIDE FRAGMENTS OF MURINE EPIDERMAL GROWTH FACTOR AS LAMININ RECEPTOR TARGETS

APPLICANT(S) FOR DO/EO/US

THE QUEEN'S UNIVERSITY OF BELFAST

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☒ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☐ Other items or information:

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 09/673785)	INTERNATIONAL APPLICATION NO. PCT/GB99/01211	ATTORNEY'S DOCKET NUMBER 41934/23838
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21. The following fees are submitted:				CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :					
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1,000.00					
<input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00					
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00					
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00					
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).				\$0.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	8 - 20 =	0	x \$18.00	\$0.00	
Independent claims	3 - 3 =	0	x \$80.00	\$0.00	
Multiple Dependent Claims (check if applicable).			<input checked="" type="checkbox"/>	\$270.00	
TOTAL OF ABOVE CALCULATIONS =				\$1,130.00	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable).				<input type="checkbox"/>	\$0.00
SUBTOTAL =				\$1,130.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).				\$0.00	
TOTAL NATIONAL FEE =				\$1,130.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).				<input type="checkbox"/>	\$0.00
TOTAL FEES ENCLOSED =				\$1,130.00	
				Amount to be: refunded	\$
				charged	\$

- ☐ A check in the amount of _____ to cover the above fees is enclosed.
- ☒ Please charge my Deposit Account No. **20-0823** in the amount of **\$1,130.00** to cover the above fees.
A duplicate copy of this sheet is enclosed.
- ☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **20-0823** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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REGISTRATION NUMBER

20 OCTOBER 2000

DATE

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PEPTIDE FRAGMENTS OF MURINE EPIDERMAL GROWTH FACTOR AS LAMININ RECEPTOR TARGETS

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2

1 cancers (breast, gastrointestinal, lung, prostatic,
2 etc), diabetic retinopathy, neovascular glaucoma,
3 rheumatoid arthritis and psoriasis. Angiogenesis is
4 the rate-limiting step in the growth of secondary
5 tumours; inhibition of their neovascularisation is
6 known to stop their growth.

7
8 In this field it is already known that the native
9 ligand of the 67kDa laminin receptor (67LR) is
10 encompassed by the linear sequence of amino acids 925-
11 933 of the laminin β -1 (previously known as laminin B1
12 or b1) chain (numbering refers to the mature murine
13 laminin β -1). Synthetic laminin β -1₉₂₅₋₉₃₃ (single letter
14 amino acid code: CDPGYIGSR-NH₂) has been shown to
15 inhibit tumour establishment in mice, by inhibiting
16 attachment of tumour cells to basement membranes. It
17 has also been demonstrated that laminin β -1₉₂₅₋₉₃₃
18 inhibits angiogenesis in the chick.

19
20 However, synthetic laminin-derived peptide (laminin
21 β -1₉₂₅₋₉₃₃) stimulates angiogenic events in mammalian
22 cells (in which it acts as a pure 67LR agonist), making
23 it useless as the basis of a human therapy.

24
25 It is one object of the present invention to provide a
26 medicament to treat angiogenic diseases.

27
28 The present invention provides a peptide factor derived
29 from murine epidermal growth factor (EGF) peptide for
30 use in the preparation of a medicament for the
31 treatment of angiogenic diseases.

32
33 The mechanism by which EGF derived peptides inhibit new
34 blood vessel formation is through their antagonism of
35 the high affinity 67 kDa laminin receptor (67LR) found
36 on endothelial cells.

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3

1 The peptides have the additional effect of inhibiting
2 tumour cell attachment to basement membranes, and may
3 be used to prevent solid cancer spread in cases where
4 cancer cells have been identified circulating in the
5 blood.

6
7 Modified peptides may be protected from proteolytic
8 degradation by substitution of key residues with
9 unnatural amino acid analogues at susceptible bonds,
10 such as tyrosine analogues (at position 5) and arginine
11 analogues (at position 9). The peptides may be capped
12 at N- and C-termini (with acetyl and amide groups
13 respectively) and at the thiol groups of the cysteines
14 (with acetamido methyl groups).

15
16 Typically the peptide is an antagonist of the 67kDa
17 Laminin Receptor (67LR).

18
19 The peptide factor is based on amino acid residues 33
20 to 42 of murine epidermal growth factor (mEGF).

21
22 The amino acid sequence of mEGF- (33-42) is CVIGYSGDRC.

23
24 Preferably the sequence of peptide factor is modified
25 from the natural sequence to protect the peptides from
26 protease attack.

27
28 Preferred substitutions include the use of tyrosine
29 analogues at position 5 and arginine analogues at
30 position 9.

31
32 Preferably the peptide factor is capped at the N
33 terminal with an acetyl group.

34
35 Preferably the peptide factor is capped at the C
36 terminal with an amide group.

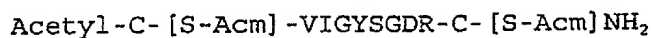
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4

1 Preferably the thiol groups of cysteines are capped
2 with acetamido methyl groups.

3
4 In one embodiment the synthetic peptide has the
5 sequence

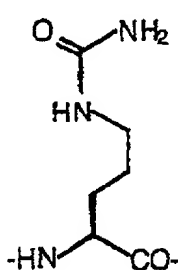


8
9 A preferred tyrosine analogue is Tic-OH.

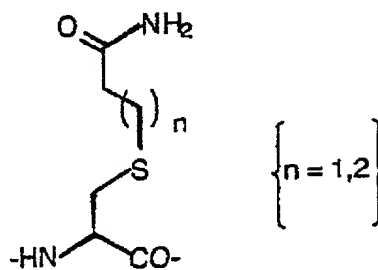
10
11 A preferred arginine analogue is Citrulline.

12
13 The structure of Citrulline and other potential
14 arginine analogues are shown below.

15
16 **Citrulline and analogues**

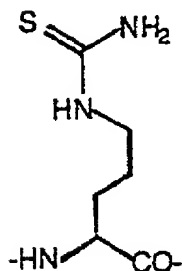


26 citrulline

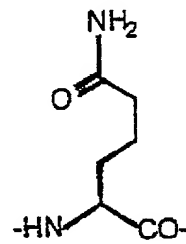


26 cysteine-derived analogues

27 {prepared by reaction of cysteine with
28 Br-(CH₂)_n-CONH₂}



thiono-citrulline



homo-glutamine

{prepared by reaction of ornithine with ammonium
isothiocyanate}

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5

1 Preferably the peptide is truncated to a shorter
2 peptide without losing its antagonistic character.

3

4 The invention further provides a peptide agonist.

5

6 The agonist may be the native sequence (single letter
7 amino acid code:CDPGYIGSR-NH₂) or may have the tyrosine
8 substituted by any of a variety of tyrosine analogues
9 such as the conformationally restricted Tic-OH or
10 2',6'-dimethyl-beta-methyl-tyrosines, 2-O-methyl and 2-
11 O-ethyl-tyrosine and the like.

12

13 The agonist may be useful in healing endothelial cell
14 wounding.

15

16 For example, corneal endothelial cells can be damaged
17 during cataract operations and this damage does not
18 self-repair because these endothelial cells do not
19 divide. Healing can only be effected by cell migration
20 and spreading, and this may be promoted by the agonist.

21

22 In order to explore possible conformations for the
23 parent mEGF₃₃₋₄₂ peptide, it was modelled using molecular
24 dynamics. Based on these conformations a strategy has
25 been predicted to provide proteolytic protection by
26 being able to identify residues that are important to
27 the maintenance of a three-dimensional conformation
28 essential for 67LR recognition.

29

30 The following is a description of some examples of
31 modifications and uses of the invention.

32

33 1. On the basis of the modelled structures, it was
34 found that the arginine residue participated in H-
35 bonding, and speculated that this charge may not
36 be important. A peptide was synthesised based on

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[illegible]

36 Figure 1a depicts a flat mount retina showing the

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7

1 effects of ROP and Figure 1b depicts a retina from
2 laminin-agonist treated mouse showing re-
3 canalisation of vessels.
4
5

6 **Treatment of Retinopathy of Prematurity (ROP)**
7

8 Severely premature babies are at risk of
9 developing retinopathies due to their being
10 exposed to high oxygen levels post-partum. This
11 life-saving intervention compensates for poor lung
12 development but has the unfortunate side-effect of
13 causing unnaturally hyperoxic conditions in the
14 retina. The direct effect of this is to remove
15 the normal hypoxic cues for endothelial migration,
16 resulting in inhibition of capillary growth and
17 vaso-obliteration. When these babies are returned
18 to room air, hypoxic stimuli are restored and
19 retinal angiogenesis is again induced. However,
20 the newly induced angiogenesis is chaotic and
21 uncontrolled, often resulting in abnormal
22 penetration of vessels into the vitreous (see
23 Figure 1a, below). It is the uncontrolled growth
24 of these blood vessels that ultimately leads to
25 loss of visual activity.
26

27 It has now been shown that laminin agonist
28 treatment can reverse the effects of both hyper-
29 oxia induced vaso-ablation as well as norm-oxia-
30 induced angiogenesis in a murine model of
31 retinopathy of prematurity (ROP). In this model,
32 development of ROP can be prevented by treatment
33 of neonates with daily injections
34 (intraperitoneal) of 10 μ g of synthetic laminin β -
35 1₉₂₅₋₉₃₃ (also referred to as laminin B1₉₂₅₋₉₃₃, single
36 letter amino acid code:CDPGYIGSR-NH₂). See Figure

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8

1 lb in comparison with 1a. Treatment with laminin agonist (Figure 1b) prevents the uncontrolled angiogenic response of ROP (Figure 1a) and promotes re-canalisation of areas of vaso-obliteration.

The invention is demonstrated with reference to the following figures wherein Figure 1a depicts a flat mount retina showing the effects of ROP

Figure 1b depicts a retina from laminin-agonist treated mouse showing re-canalisation of vessels.

Murine model of proliferative retinopathy

Litters of 7 day old C57-BL/6J mice, together with their nursing dams, are exposed to 80% oxygen in an incubator maintained at 23°C and with a gas exchange of 1.5L/min for 5 days according to the protocol described by Stitt et al. (1998). On postnatal day 12 (P12) the animals are returned to room air and sacrificed at various times post-hyperoxia. Animals are treated with daily i.p. injections of either laminin agonists (10µg per head per day) or vehicle control. Groups of room air controls are maintained in parallel with hyperoxia-exposed animals. Home Office project and personal licenses are held for this work. All animals are housed and maintained in accordance with the ARVO regulations for animal care in research.

Animals are sacrificed at pre-determined key stages in the vaso-obliteration (P7-P12), ischaemia (P12 onwards) and vaso-proliferative responses (P12-21). At sacrifice, terminally

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9

1 anaesthetised animals have a single eye enucleated
2 and the retina removed to be snap-frozen for later
3 RNA-extraction (see below). The fellow eye is
4 either perfused with fluorescein dextran or
5 enucleated and fixed in 4% paraformaldehyde for
6 histology, immunohistochemistry and *in situ*
7 hybridisation.

8

9 ALTERNATIVE USES

10

11 1. Treatment of corneal wounds

12

13 The cornea is a delicate transparent structure.
14 Being avascular, corneal wound healing depends
15 upon local self-renewal of the corneal epithelium.
16 This, in turn, depends upon the presence of a
17 mitogenically functional stem cell population
18 ('limbal cells'), which produce replacement cells
19 that migrate and desquamate at the denuded area.
20 Damage to these underlying stem cell populations
21 causes inappropriate re-epithelialization by
22 conjunctival cells followed by matrix deposition
23 and scar formation. The damaging agent may be
24 corrosive chemical or heat burns, erosion by
25 contact lenses, Stevens Johnson disease.

26

27 It is known that transplantation of limbal cell
28 autografts from the unaffected eye can restore a
29 stable healing of the corneal epithelium (Kenyon
30 et al., 1996). It has been proposed that
31 harvesting small samples of limbal stem cells,
32 followed by serial culture *in vitro* would provide
33 greater chance of success (particularly when both
34 eyes are affected) De Luca, et al., 1997).
35 However, with both protocols, correct uptake and
36 controlled migration of these grafted cells into

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10

1 the corneal epithelium has not been optimised.

2

3 We propose that laminin agonists could be used to
4 stimulate the migratory response of the cells
5 prior to grafting, or alternatively topical
6 application of laminin agonists to the wound site
7 could be used to direct migration of the grafted
8 cells to the correct (denuded) area of the cornea.

9

10 2. Some microbial pathogens such as *Candida albicans*,
11 express 67LR and use this as a means of attaching
12 to human basement membranes. It is conceivable
13 that such infections could be abolished by
14 treatment with mEGF₃₃₋₄₂-derived peptides, which
15 would prevent the microbes from adhering to the
16 host.

17

18 EXAMPLE 2

19

20 Peptide Study

21

22 The purpose of the investigation was to determine the
23 molecular target of mEGF₍₃₃₋₄₂₎ and to identify the amino
24 acids that are essential for receptor recognition. In
25 addition, the key residues which confer laminin
26 antagonism on mEGF₍₃₃₋₄₂₎ were examined.

27

28 Two lead compounds were investigated; synthetic laminin
29 β -1 sequence CDPGYIGSR-NH₂ and mEGF₍₃₃₋₄₂₎ sequence
30 AcC(Acm)-VIGYSGDRC-(Acm)-NH₂. Bearing in mind the pure
31 antagonism of the murine EGF peptide, the aims of this
32 study were to identify the key residues responsible for
33 these contrasting activities using alanine scanning, in
34 the context of developing anti-angiogenic drugs for
35 retinopathy treatment.

36

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11

1 In addition, using residue exchange between the two
2 peptides and molecular modelling to predict three-
3 dimensional structure, we wished to further investigate
4 the role of individual mEGF₍₃₃₋₄₂₎ residues in laminin
5 antagonism. A logical series of peptides was
6 synthesised and screened for receptor interaction, cell
7 adhesion and motility properties (Table 1a and 1b).

8

9 MATERIALS AND METHODS

10

11 Peptide synthesis

12

13 Peptide sequences based on and mEGF₍₃₃₋₄₂₎ were
14 synthesised on a model 432A peptide synthesizer
15 (Applied Biosystems, Warrington, UK), using standard
16 solid-phase Fmoc procedure (Fields 1990). Synthesis of
17 the peptides required successive additions of
18 derivatized amino acids to form a linear product.

19

20 Peptides were purified after synthesis using reverse
21 phase HPLC and purity confirmed by automated amino acid
22 analysis and electrospray mass spectrometry. All
23 peptide sequences were stored in the presence of
24 desiccant at -20°C until required for biological assay.

25

26 Laminin receptor antibody production

27

28 a. Preparation of MAPs

29

30 The peptide sequence (PTEDWSAQPATEDWSAAPT),
31 corresponding to the COOH-terminal end of the human
32 laminin receptor, was used as the antigen template.
33 Derivation of the peptide, based on a CN-Br cleavage
34 fragment of the cDNA sequence encoding human laminin
35 receptor, has been described elsewhere (Wewer et al
36 1986). The antigen was synthesised as an octomeric

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12

1 peptide derivative (MAPs) using automated Fmoc
 2 procedure (Tam 1988).

3

4

5 Table 1a: Peptide substitution

mEGF ₍₃₃₋₄₂₎	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys-NH ₂
I	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ile	Gly	Asp	Arg	ACM Cys-NH ₂
II	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Ser	Arg	ACM Cys-NH ₂
III	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ile	Gly	Ser	Arg	ACM Cys-NH ₂
IV	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Clt	ACM Cys-NH ₂
V	acetyl	ACM Cys	Val	Ile	Gly	OH Tic	Ser	Gly	Asp	Arg	ACM Cys-NH ₂

Table 1b: Peptide substitution (alanine scanning)

mEGF ₍₃₃₋₄₂₎	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys- NH ₂
VI	acetyl	ACM Cys	Val	Ala	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys- NH ₂
VII	acetyl	ACM Cys	Ala	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys- NH ₂
VIII	acetyl	Ala	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys- NH ₂
IX	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	Ala- NH ₂
X	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ala	Gly	Asp	Arg	ACM Cys- NH ₂

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13

1 b. Immunisation schedule

2
3 A pre-immune test bleed (5ml) was obtained from the
4 marginal ear vein of a male New Zealand White rabbit
5 (3.2 kg). The bleed was allowed to clot for 2 h at room
6 temperature after which its edge was detached from the
7 wall of the collection vessel. The clot was then
8 allowed to contract overnight at 4°C. Serum was then
9 removed and the residual material pelleted out by
10 centrifugation (10 min at 2,500 g). Extracted serum
11 (3.5 ml) was then frozen at -20°C until required.

12
13 Immunogen was prepared by the emulsion of MAPs (0.5 g
14 antigen in 0.5 ml PBS) in an equivalent volume of
15 adjuvant (Alum Imject; Pierce, Chester, UK). The
16 animals immune system was primed by introducing
17 immunogen (50 µg) through subcutaneous injection at
18 different sites on the animals back. The rabbit was
19 boosted by both subcutaneous and intramuscular
20 injection, 21 days after priming, using an increased
21 dose of immunogen (800 µg). Subsequent boosts were
22 performed by intramuscular injection after a further 14
23 days (800 µg immunogen), and thereafter at 21 day
24 intervals. Test bleeds were taken 2 days after each
25 boost and the serum extracted as described above. The
26 animal was boosted and bled a total of three times.

27
28 c. Enzyme-linked immunoabsorbent assay

29
30 ELISA was used to determine the specificity of the
31 antibody prepared against the synthetic MAPs peptide
32 and to determine the efficacy of binding with respect
33 to that of the linear precursor.

34
35 Peptides were dissolved in distilled water and diluted

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14

1 to 10 $\mu\text{g/ml}$ in coating buffer. Aliquots (100 μl) of
2 either linear or MAPs peptide were then added to the
3 wells of microtitre plates (Microtest III; Becton
4 Dickinson Ltd., Oxford, UK) and incubated overnight at
5 37°C. The wells were then rinsed with 100 μl wash
6 buffer and air dried. Excess adsorption sites were
7 blocked (1 h incubation at 22°C) by the addition of 10%
8 casein in PBS (0.1 ml/well). Subsequent to the removal
9 of casein solution by aspiration, wells were again
10 rinsed with wash buffer and air dried.

11
12 Antisera or pre-immune sera were then serially diluted
13 in PBS and 100 μl of each incubated in peptide coated
14 wells for 1 h at 37°C. After rinsing (0.1 ml wash
15 buffer), 100 μl per well of 5 $\mu\text{g/ml}$ secondary antibody
16 (horse-radish peroxidase-conjugated goat anti-rabbit
17 IgG; Amersham International, Aylesbury, UK) was added
18 to each well and the plates incubated at 37°C for 1 h.

19
20 Wells were again rinsed with wash buffer and 0.1 ml
21 substrate solution (TMB peroxidase) added to each. The
22 plate was then incubated at 22°C for 30 min and the
23 colour reaction stopped by the addition of 0.5M H_2SO_4
24 (0.1 ml/well). Absorbance was measured at 450 nm on a
25 Titertek Multiscan plate reader.

26 27 d. Purification of IgG fraction

28
29 Anti-laminin receptor antiserum was purified using
30 immobilised protein G-sepharose columns (Pharmacia
31 Biotech, Uppasla, Sweden). The columns were
32 equilibrated with 20 ml sodium phosphate buffer (pH
33 7.0). Antiserum was diluted 1:4 in the same buffer and
34 a 1 ml aliquot loaded onto the column (flow rate 150
35 ml/h, fraction size 2.5 ml). After exclusion of the

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15

1 unbound fraction, as determined by absorbance at 280nm,
2 the IgG component of the antiserum was eluted with 0.1M
3 glycine-HCl (pH 2.7), into tubes containing 0.1 ml Tris
4 (1M), pH 9.0. The eluted IgG fractions were bulked and
5 stored at -20°C until required.

6

7 Maintenance of cell cultures

8

9 Cancer and endothelial cells were maintained in either
10 DMEM (T47-D) or RPMI (SK HEP-1) media, supplemented
11 with 10% FCS, 100 IU/ml penicillin and 100 µg/ml
12 streptomycin. Cells were incubated at 37°C in a
13 humidified atmosphere of 95% air: 5% CO₂ and media
14 refreshed as required. Cultures (at 80-85% confluence)
15 were routinely passed on removal from monolayer by the
16 action of trypsin (0.25%) and EDTA (0.02%) in CFS.

17

18 The viability of cell populations following
19 trypsinisation was determined by the trypan blue vital
20 dye exclusion test. Populations confirmed as being in
21 excess of 95% viable were used in all studies.

22

23 Media were screened for possible bacterial or fungal
24 contamination by incubating 1ml aliquots with both
25 nutrient and Saboraud dextrose broths (Oxoid Ltd.,
26 Basingstoke, UK). Cell populations were routinely
27 monitored for sub-clinical infections by periodically
28 culturing in the absence of antibiotics.

29

30 Both cell lines and media were examined for the
31 presence of contaminating *Mycoplasma* spp. by the method
32 of Chen (1977).

33 Determination of cell numbers

34

35 Single cell suspensions were quantified using an

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1 automated counter (Coulter Electronics, Harpenden, UK).
2 A 1 ml aliquot of cell suspension was diluted 1 in 20
3 in Isoton and 0.5 ml samples counted. The mean of 5
4 counts was taken and the total number of cells
5 determined. Estimates of cell number were confirmed by
6 counting in a haemocytometer.

7
8 For microtitre end-point assays, cell numbers were
9 estimated from the crystal violet staining index of the
10 cell line (Kanamaru and Yoshida 1989). Briefly, after
11 removal of media from the assay system cells were fixed
12 with formaldehyde (10% in PBS), and washed with
13 distilled H₂O. Aliquots (100 μ l) of crystal violet
14 solution (0.1% in distilled H₂O) were added to each well
15 and the plates allowed to stand for 30 min. Excess
16 stain was removed by rinsing with distilled H₂O (3 x 100
17 μ l). The wells were then air-dried and the remaining
18 crystal violet extracted with 100 μ l acidified
19 methanol. Absorbance at 620 nm was determined using a
20 Titertek Multiscan spectrophotometer.

21 22 Proliferation assays

23
24 The effects of synthetic peptides and growth factors on
25 the growth of breast cancer and endothelial cells were
26 determined as detailed.

27
28 Exponentially growing cells were harvested by
29 trypsinisation, as previously described. After rinsing
30 and resuspending in the relevant culture media
31 (containing 10% FCS), the cells (100 μ l aliquots) were
32 dispensed into 96-well microtitre plates at a
33 population density of 2×10^4 cells/well (6 wells per
34 experimental condition). Cells were incubated for
35 24 h at 37°C after which the media was removed and the

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1 wells rinsed with CFS (3 x 100 μ l), to rid the plates
2 of cells in suspension. Media was then replaced with
3 that containing the relevant controls or treatment
4 supplements as detailed in individual experiments.

5
6 Cell numbers were evaluated spectrophotometrically at
7 620 nm, over the period of assay, after fixing with 10%
8 formaldehyde and staining with crystal violet.

9
10 Proliferative responses were analysed using the
11 Wilcoxon Rank test and significant differences at the p
12 < 0.05 level, defined. Results of all growth studies
13 were confirmed in at least 3 individual experiments.

14
15 **Laminin attachment assay**

16
17 Non-tissue culture grade 96-well plates, coated with
18 2.5 μ g murine laminin in 50 μ l CFS per well, were air-
19 dried overnight at room temperature. Preliminary
20 experiments indicated that cell attachment was
21 concentration dependent; maximal binding occurred at a
22 laminin coating of 2.5 μ g/well. After rinsing with CFS
23 (100 μ l), the plastic was saturated with casein (0.2%
24 in CFS). Plates were incubated at room temperature for
25 45 min then washed extensively with CFS (3 x 100 μ l).

26
27 After removal of culture media, cells were detached
28 from monolayers by the action of EGTA (0.02% in CFS) at
29 37°C. The cells were then centrifuged at 800 g for 2
30 min and the pellet resuspended in DMEM (T-47D) or RPMI
31 (SK HEP-1).

32
33 Cells, at a population density of 10^6 cells/ml, were
34 then aliquoted (1 ml) into microfuge tubes containing
35 the individual peptide sequences and incubated for 1 h

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1 at 37°C. The cells (100 μ l aliquots) were then added to
2 the pre-coated multi-well plates and incubated for a
3 further 60 min. Incubation media were removed and the
4 wells washed with CFS (3 x 100 μ l) to rid the plates of
5 non-adherent cells.

7 Attached cell numbers were evaluated
8 spectrophotometrically at 620 nm after fixing with 10%
9 formaldehyde and staining with crystal violet.

11 Attachment to mEGF₍₃₃₋₄₂₎

13 That mEGF₍₃₃₋₄₂₎ bound to the 67kDa laminin receptor was
14 demonstrated using a biotinylated derivative of the
15 peptide (Acetyl-C-[S-Acm]-VIGYSGDR-C-[S-Acm]-K-[N^ε-
16 biotin]-amide) and a modification of the above laminin
17 attachment assay.

Briefly, 96-well plates were coated with 100 μ l/well streptavidin (5 μ g/ml in carbonate buffer pH 9.6) and following an overnight incubation at 37°C, wells were washed with CFS (3 x 100 μ l) and the plastic blocked with casein (0.2% in CFS). The plates were then incubated at room temperature for 45 min and washed with CFS as previously detailed. Biotinylated mEGF₍₃₃₋₄₂₎ in CFS was then aliquoted into the wells (0.1 ml of 100 μ M) and the plates incubated for 3 h at 37°C.

29 After a further block with 0.2% casein, the wells were
30 washed with CFS (3 x 100 μ l aliquots). Plates were kept
31 at 4°C and used within 2 h.

33 Cells were prepared as above and pre-incubated for 1 h
34 at 37°C with serial dilutions of anti-laminin receptor
35 polyclonal (see below) or anti-EGF (R1) receptor

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19

1 monoclonal antibodies. Subsequent procedures were as
2 detailed for the laminin attachment assay.

3

4 Laminin receptor binding determinations

5

6 a. Radiolabelling of laminin

7

8 ^{125}I -laminin was prepared using ^{125}I -labelled sodium
9 iodide (Amersham, UK) and immobilised chloramine-T
10 (Iodobeads; Pierce, Illinois). Prior to use, the beads
11 were washed with 500 μl phosphate buffer (pH 6.5) to
12 remove excess reagent from the support. These were then
13 allowed to air dry and individual beads added to a
14 solution of carrier free Na^{125}I , diluted with iodination
15 buffer (phosphate buffer pH 7.4). The beads were
16 allowed to equilibrate for 5 min.

17

18 Laminin (10 μg in 10 μl) was then diluted into the
19 iodination buffer and the system incubated at 20°C for
20 15 min. The solution was then removed from the reaction
21 vessel and excess Na^{125}I and unincorporated $^{125}\text{I}_2$,
22 separated from the iodinated protein by gel filtration
23 on a GF-5 exclusion column (Pierce, Illinois).
24 Iodinated laminin fractions were recovered at a
25 specific activity of approximately 1.2 mCi/mg protein
26 (864 Ci/mmol).

27

28 b. Competition binding estimation

29

30 Near confluent cultures of T47-D or SK HEP-1 cells were
31 removed from monolayer with 0.02% EGTA and passed
32 through a G-25 syringe needle to produce single cell
33 suspensions. Aliquots of each cell type (10^6 cells/ml)
34 were dispensed into separate Ependorf tubes (1 ml each)
35 and pelleted. The cells were then resuspended in 1 ml

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20

1 ice-cold RPMI (SK HEP-1) or DMEM (T47-D) containing
2 0.1% BSA and either laminin or synthetic peptide at the
3 concentrations indicated. Iodinated laminin was then
4 added to each cell suspension to give a final ^{125}I -
5 laminin concentration of 0.1 nM (approximately 50,000
6 cpm). These mixtures were incubated overnight at 4°C.

7
8 The tubes were then microfuged at 10,000 g and the
9 supernatant removed. After washing the pellet with 500
10 μl CFS, the remaining radioactivity was determined
11 using a gamma radiation counter. Non-specific binding
12 was determined by incubating cells with a 1000-fold
13 molar excess of unlabelled laminin. All estimations
14 were carried out in triplicate.

15
16 IC_{50} (concentration of unlabelled peptide required to
17 produce 50% inhibition of radioligand binding) and EC_{50}
18 (effective concentration for 50% inhibition of cell
19 attachment) values were calculated using the Grafit
20 curve-fitting programme (Erithacus Software, London,
21 UK).

22 23 Migration assays

24
25 The method used was basically as described by Albrecht-
26 Buehler (1977). Briefly, coverslips (22 x 22 mm) were
27 treated in 5% detergent (7X; ICN Biomedicals) and
28 washed in alcohol to remove grease. After drying, they
29 were immersed in gelatin solution (Sigma, 300 Bloom;
30 0.5 g in 300 ml distilled H_2O) for 10 min. The
31 coverslips were then dried by placing in a 70°C oven
32 for 45 min.

33
34 Colloidal gold suspension was prepared by adding 11 ml
35 distilled H_2O and 6 ml Na_2CO_3 (36.5 mM) to 1.8 ml AuHCl_4

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1 (14.5 mM). The mixture was heated to 95°C at which
2 point 1.8 ml of freshly prepared 0.1% formaldehyde
3 solution was added; the temperature was maintained at
4 95°C. A suspension of colloidal gold was formed which
5 was brown to absorbed light and blue to transmitted
6 light.

7
8 The gold suspension, was then added to petri dishes
9 containing individual coverslips and the plates
10 incubated at 37°C for 45 min. After washing with CFS (3
11 x 4 ml) to remove unattached gold particles, the
12 coverslips were transferred to 6-well cluster dishes
13 and UV sterilised.

14
15 Endothelial cells (SK HEP-1 and BRCE) in culture media
16 (0.3 ml) were seeded onto the coverslips at an
17 approximate density of 5×10^3 cells per well. The cells
18 were allowed to plate down for 2 h at 37°C after which
19 the treatments were added. Assay systems were
20 maintained for a further 18 h after which the cells
21 were fixed using 3% gluteraldehyde in cacodylate buffer
22 (pH 7.2).

23
24 The assays were examined using a Leica DM1RB phase
25 contrast microscope and Q500MC image analysis system
26 incorporating a JVC TK-1280E colour camera (Leica,
27 Milton Keynes, UK). The track images of at least 30
28 cells were video-captured and the area (representing
29 migration response) determined for each. Statistical
30 analysis of these areas was then carried out using
31 Macintosh Instat software to perform both Kruskal-
32 Wallis analysis of variance and Mann-Whitney U-tests in
33 order to compare the treatment groups with controls.

34

35 RESULTS

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2

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21

27

28 In addition, it is shown that the commonly used chick
29 angiogenesis models are not appropriate to the study of
30 laminin mediated human angiogenesis: although it is
31 confirmed that Lam. β -1₍₉₂₅₋₉₃₃₎ acts as a partial laminin
32 antagonist in chick, it was found to be a pure agonist
33 in mammalian cell lines. This is a highly significant
34 point given that pharmaceutical companies (such as
35 Anqiotech, Vancouver, BC) are using the chick CAM assay

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23

1 as the sole screening method for the discovery of anti-
2 angiogenic lead compounds. This may be inappropriate
3 for use in human disease.

4
5 This study is the first to show that the YIGSR-receptor
6 is, in fact, the 67 kda high affinity laminin receptor
7 (67-LR). In collaboration with Professor Archer's team
8 at the Department of Ophthalmology, Royal Victoria
9 Hospital, Belfast, it has been determined that the 67-
10 LR is preferentially expressed in new vessels during
11 oxygen-induced retinopathy in neonatal mice.

12
13 **Peptide antagonist development**

14
15 The *N*-terminus of Lam. β -1₍₉₂₅₋₉₃₃₎ is not necessary for
16 receptor recognition and the agonist activity of YIGSR
17 peptide (Ostheimer et al 1992, Kawasaki et al 1994).

18
19 However, alanine scanning of the starting peptide
20 (mEGF₍₃₃₋₄₂₎) indicated that residues at positions 1, 2,
21 3, and 6 (peptides VI, VII, VIII and X respectively),
22 are essential for receptor mediated activities as
23 determined by ¹²⁵I-laminin displacement and cell
24 attachment to laminin through the 67-LR. Substitution
25 of these individual residues by alanine leads to a
26 dramatic decrease in receptor affinity observed as an
27 increased IC₅₀ (Table 2) and a parallel decrease in
28 their ability to block adhesion to laminin (increased
29 EC₅₀; Table 2). Characterisation of these analogues with
30 regard to effects on motility, largely confirmed these
31 findings although there was one exception; peptide
32 VIII. Results from the migration assay identified this
33 sequence (alanine for cysteine (P1)) as being a weak
34 laminin agonist despite there being a much reduced
35 response in the other two assays. It is suggested that

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1 this peptide may influence laminin receptor mediated
2 migration through an alternative mechanism (Scott
3 1997).

4
5 Substitution at P10 (alanine for cysteine (peptide X)
6 retains both receptor binding and adhesion displacing
7 activities but has the effect of changing the
8 antagonistic parent into an agonist analogue. This
9 reflects the response the agonism of Lam. β -1₍₉₂₅₋₉₃₃₎,
10 which also lacks the C-terminal cysteine, and suggests
11 that this cysteine is not essential for receptor
12 recognition, but is required for antagonism of mEGF₍₃₃₋₄₂₎.

13
14
15 Studies have reported that the positive charge offered
16 by arginine (P9) is essential for the biological
17 activity of Lam. β -1₍₉₂₅₋₉₃₃₎ (McKelvey et al 1991, Kawasaki
18 et al 1994). Glutamate substitution for arginine
19 generates a negative charge at this position with
20 corresponding loss of biological activities (Kawasaki
21 et al 1994).

22
23 However, the substitution of arginine (P9) with
24 positively-charged lysine (McKelvey et al 1991) also
25 results in complete loss of ligand binding and
26 biological activities, suggesting that the mere
27 presence of a positive charge at this position is, in
28 itself, insufficient for receptor recognition. This
29 modelling studies suggest that H-bonding of the
30 guanidino group of the arginyl residue to the aromatic
31 sidechain of the tyrosyl residue (P5) in the consensus
32 sequence GYXGXR presents an acceptable motif for 67-LR
33 activation by both mEGF₍₃₃₋₄₂₎ and Lam. β -1₍₉₂₅₋₉₃₃₎.

34
35 Substitution of tyrosine (P5) with a conformationally

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25

10

14

25

33

34 Substitution of isoleucine (P6) for serine (peptide I)
35 resulted in both reduced receptor affinity and potency

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26

1 in displacement of cell adhesion to laminin. However,
2 this analogue retained weak antagonist activities in
3 the motility assay. It is therefore of interest that
4 studies on the YIGSR sequence indicate that residue
5 substitution, at the position taken by isoleucine in
6 the pentapeptide, are well tolerated and may increase
7 potency (Kawasaki et al 1994).

8
9 Replacement of aspartate (P8) with serine (peptide II)
10 resulted in a complete loss of biological function. as
11 did peptide III encompassing both the former
12 (isoleucine (P6) for serine) and latter (serine (P8)
13 for aspartate) substitutions. Since this mEGF₍₃₃₋₄₂₎
14 analogue sequence (peptide II) encompasses the active
15 YIGSR amino acid sequence agonist, it is suggested that
16 this loss of activity may be attributed to the valine
17 (P2) and isoleucine (P3) residues in the N-terminal
18 half of mEGF₍₃₃₋₄₂₎. Alternatively, addition of a C-
19 terminal cysteine to the YIGSR sequence is known to
20 reduce potency (Kawasaki et al 1994). Additional
21 peptides incorporating the valine (P2) and isoleucine
22 (P3) substitutions are currently under investigation.

23
24 The determination of the minimum core peptide structure
25 is ongoing and involves similar characterisation
26 studies on a number of sequences truncated at the C-
27 terminal.

28
29 These studies have thus identified an important
30 antagonist of 67-LR mediated activities in peptide IV.
31 The sequence, (AcC(Acm)-VIGYSGD-[Cit]-C-(Acm)-NH₂), may
32 provide an important template for anti-angiogenic drugs
33 in that it is resistant to cleavage by trypsin-like
34 proteases and has been identified as being more potent
35 than mEGF₍₃₃₋₄₂₎ in screening procedures.

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27

1 Advantages

2

3 The advantages of the invention, and the ways in which
4 disadvantages of previously known arrangements are
5 overcome include:

6

7 1. Unlike the native 67LR ligand (laminin β -1₉₂₅₋₉₃₃),
8 which is angiogenic in human models, the mEGF₃₃₋₄₂-
9 derived agents are anti-angiogenic in human
10 models.

11

12 2. mEGF₃₃₋₄₂ has the advantage of inhibiting both
13 laminin- and EGF-stimulated angiogenesis.

14

15 3. mEGF₃₃₋₄₂ prevents tumour cell attachment to
16 basement membranes.

17

18

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1 CLAIMS

2

3 1 A peptide factor derived from amino acid residues 33
4 to 42 of murine epidermal growth factor peptide or a
5 synthetic equivalent thereof wherein the peptide
6 factor is modified to protect it from proteolytic
7 degradation and the peptide binds to laminin
8 receptors wherein the modifications consist of at
9 least one modification chosen from the group
10 comprising; substitution of tyrosine by tyrosine
11 analogues and substitution of arginine by arginine
12 analogues,
13

14 2 A peptide as claimed in Claim 1 wherein the
15 peptide is further modified by at least one
16 modification chosen from the group comprising
17 capping the N terminal of the peptide, capping
18 the C terminal of the peptide and capping thiol
19 groups of cysteines.
20

21 3 A peptide as claimed in claims 1 or 2 wherein
22 tyrosine is substituted by Tic-OH.
23

24 4 A peptide as claimed in claims 1, 2 or 3 wherein
25 arginine is substituted by Citrulline.
26

27 5 Use of a peptide factor as claimed in any of the
28 preceding claims in the preparation of a medicament
29 to bind laminin receptors as an antagonist.
30

31 6 Use of a peptide factor as claimed in any of the
32 preceding claims in the preparation of a medicament
33 to bind laminin receptors as an agonist.
34

35 7 Use as claimed in claim 6 in the preparation of a
36 medicament for healing endothelial cell wounding.

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1 8 Use as claimed in claim 8 or 9 for the treatment of
2 retinopathy of immaturity.

[illegible]

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Fig 1a

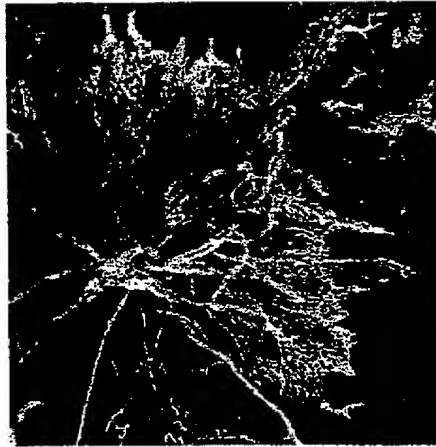
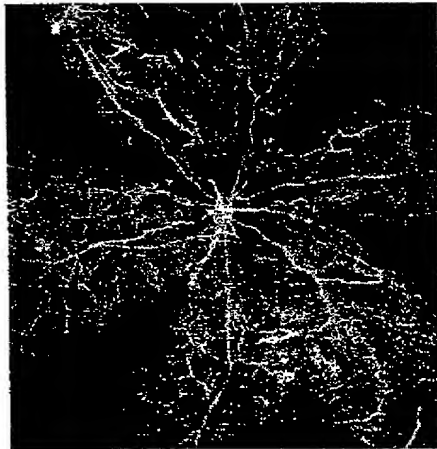


Fig 1b



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41934/23838

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PEPTIDE FRAGMENTS OF MURINE EPIDERMAL GROWTH FACTOR AS LAMININ RECEPTOR TARGETS

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on 21.04.99 as United States Application No. or PCT International

Application Number PCT/GB99/01211

and was amended on 16.06.2000

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
<u>9808407.2</u>	<u>GB</u>	<u>22.04.98</u>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	
<u> </u>	<u> </u>	<u> </u>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	
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(Number)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

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(Application Serial No.)	(Filing Date)
_____	_____
(Application Serial No.)	(Filing Date)
_____	_____
(Application Serial No.)	(Filing Date)

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_____	_____	_____
PCT/GB99/01211	21.04.99	PENDING
(Application Serial No.)	(Filing Date)	(Status)
		(patented, pending, abandoned)
_____	_____	_____
(Application Serial No.)	(Filing Date)	(Status)
		(patented, pending, abandoned)
_____	_____	_____
(Application Serial No.)	(Filing Date)	(Status)
		(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

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